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Mutation of the Androgen Receptor at Amino Acid 708 (Gly→Ala) Abolishes Partial Agonist Activity of Steroidal Antiandrogens

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ABSTRACT

Mutation of a single amino acid in the ligand-binding domain (LBD) of the human androgen receptor (hAR) can induce functional abnormalities in androgen binding, stabilization of active conformation, or interaction with coactivators. The Gly708Ala and Gly708Val substitutions are associated with partial and complete androgen insensitivity syndromes, respectively. In this work, we introduced Ala, Val, and aromatic Phe mutations at position 708 on helix H3 of the hAR-LBD and tested the functional and structural consequences on hAR activity in the presence of steroidal or nonsteroidal agonists and antagonists. The residues involved in the specific recognition of these androgen ligands were identified and analyzed in the light of in vitro biological experiments and the 3D hAR-LBD structure. Our study demonstrated that the Gly708Ala mutation influenced the agonist versus antagonist activity of the ligands and confirmed the crucial role of this residue within the ligand-binding pocket (LBP) in the modulation of androgen agonists. The Gly708Ala mutation transformed the antiandrogen cyproterone acetate (CPA), a partial agonist, into a pure antiandrogen, and the pure nonsteroidal antiandrogen hydroxyflutamide in a partial agonist. From the docking studies, we suggest that CPA acts on AR through the novel mechanism called "passive antagonism".

The androgen receptor (AR) belongs to a steroid nuclear receptor superfamily of ligand-activated transcription factors that includes the other steroid receptors and thyroid hormone, vitamin D3 and retinoic acids, and orphan receptors whose ligands have not yet been identified (Evans, 1988; Mangelsdorf et al., 1995). As with other members of this family, AR is characterized by three basic functional domains, including two transactivation functions (AF1 and AF2), a well conserved DNA-binding domain, and a ligand-binding domain (LBD). AR function is required for regulating male reproductive system development (Quigley et al., 1995). Mutations in the human AR (hAR) gene can alter receptor function, leading to several disease states, such as the androgen insensitivity syndrome (AIS) and prostate cancer (Gotlieb et al., 1998). Its transcriptional activity is regulated by its ligands, which may be agonists or antagonists. Testosterone and dihydrotestosterone (DHT), the two predominant natural androgens, are mediated through the AR. Interestingly, antagonists for this receptor are not closely related in structure. Antagonist steroidal synthetic compounds such as cyproterone acetate (CPA) and nonsteroidal compounds such as hydroxyflutamide, which block the actions of androgens, have proved useful in the treatment of benign prostatic hypertrophy and prostate cancer in men and hirsutism in women (McLeod, 1993).

We recently showed on the human mineralocorticoid receptor (hMR) that synthetic C-11–substituted spirolactones displayed antagonist properties but acted as potent agonists when Ala773 (helix H3) was substituted with Gly (Auzou et al., 2000). Homology modeling of the hMR LBD (Fagart et al., 1998) revealed that the Gly-for-Ala substitution in the ligand-binding pocket (LBP) explained the ability of the mutant to accommodate the bulky C-11 substituents that the

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ABBREVIATIONS: AR, androgen receptor; LBD, ligand binding domain; hAR, human androgen receptor; AIS, androgen insensitivity syndrome; DHT, dihydrotestosterone; CPA, cyproterone acetate; hMR, human mineralocorticoid receptor; LBP, ligand binding pocket; R1881, methyltrienolone; MGA, megestrol acetate; CMA, chlormadinone acetate; wt, wild type; MMTV, murine mammary tumor virus; CMV, cytomegalovirus; DCC, dextran-coated charcoal; FCS, fetal calf serum; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; GA1, 11β-vinyl-3-oxo-19-nor-17α-pregna-4,9-diene-21,17-carbolactone.

wild-type receptor cannot. These same compounds were demonstrated to be potent hAR agonists in vitro (Nirdé et al., 2001). Moreover, hAR has a glycine in the place of Ala at the corresponding position (Gly708). This region was shown to be crucial for the stabilization of the active hAR conformation, but the nature of the ligand influences its agonist/antagonist activity. This Gly708 residue, when substituted with alanine, is associated with partial AIS (Hiort et al., 1994; Albers et al., 1997) and, when substituted with Val, is associated with complete AIS (Auchus et al., 1995).

In this study, we introduced several mutations at position 708 (Ala, Val, Phe) of the hAR and tested the functional consequences of these changes on hAR activity in the presence of steroidal and nonsteroidal agonists and antagonists (Fig. 1). We provide evidence that a single amino acid substitution (Gly708Ala) caused the conversion of CPA, an antagonist with partial agonist activity, to a pure antiandrogenic ligand, and of hydroxyflutamide, a pure nonsteroidal antiandrogen, to a partial agonist. The experimental data were associated with structural analysis using the hAR-LBD crystal structure in complex with the natural agonist ligand DHT, or the synthetic one, R1881 (Matias et al., 2000; Sack et al., 2001).

Herein, we show the role of the bulky 1,2 cyclopropane ring and the chlorine on the C-6 position, as well as that of the 4,5–6,7 double bonds in the loss of anchoring of CPA, megestrol acetate (MGA), and chlormadinone acetate (CMA), by comparison with progesterone deprived of such substituents.

Compound GA1, a C-11 substituted steroid with a C-17γ lactonic ring that has already been tested on hMR and hAR (Auzou et al., 2000; Nirdé et al., 2001), was also assayed in this work for its capacity to bind and stimulate transcription of G708A hAR.

Together, these results reveal that Gly708 is a crucial component of the hAR-LBP, because the mutation of this residue modulates the agonist/antagonist behavior of AR ligands. The involvement of the region surrounding Gly708 in the process of providing active or inactive conformation of the receptor opens new directions for the design of selective androgen agonists or antagonists.

Materials and Methods

Chemicals. [^{3}H]R1881 (87 Ci/mmol) and unlabeled R1881 were purchased from PerkinElmer Life Science Products (Paris, France). 11β-Vinyl-3-oxo-19-nor-17α-pregna-4,9-diene-21,17-carbolactone (GA1; Fig. 1), described previously by Nickisch et al. (1985), was synthesized in our laboratory (Faraj et al., 1990; Claire et al., 1993). Progesterone, CPA, MGA, and CMA were from Sigma (Saint Quentin Fallavier, France). Hydroxyflutamide and bicalutamide (Casodex) were a gift from Theramex (Monaco). Nilutamide was a gift from Aventis (Strasbourg, France).

Plasmid Construction. A large C terminal portion of the androgen receptor (amino acids 387–919) containing the functional LBD was inserted into the pUC19 vector (Roche Molecular Biochemicals, Meylan, France) to generate pUC19-hAR-ΔNH$_2$. The hAR mutations (G708A, G708F, G708V) were produced by polymerase chain reaction-based point mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Sense primers were designed both to introduce the expected mutation and to create (or abolish) an enzymatic digestion site. The primer pairs used were A1 (for G708A mutation; 5′gcctc aatga actgg ccgag agaca gcttg tac 3′) and A2 (the corresponding reverse complementary primer), which generated an *EaeI* site and prevented the *Bsr*I site. The F1 sense primer was 5′gcctc aatga actgt tcgag agaca gcttg tac 3′ with F2 as the reverse complementary primer, which generated a *Taq*I site and removed the *Bsr*I site. The V1 sense primer was 5′gcctc aatga actgg tcgag agaca gcttg tac 3′ with V2 as the reverse complementary primer, which generated a *Taq*I site and prevented the *Bsr*I site. The mutated pUC-hAR-ΔNH$_2$ was subjected to Tth111I/Bst-BI digestion; this restriction fragment was agarose-purified and ligated into a pSG5-hAR wild-type (wt) vector lacking the *Tth*111I/*Bst*BI fragment to generate pSG5-hAR G708 mutants. DNA sequences encompassing the *Tth*111I and *Bst*BI sites were performed using a LICOR 4200 automated DNA sequencer

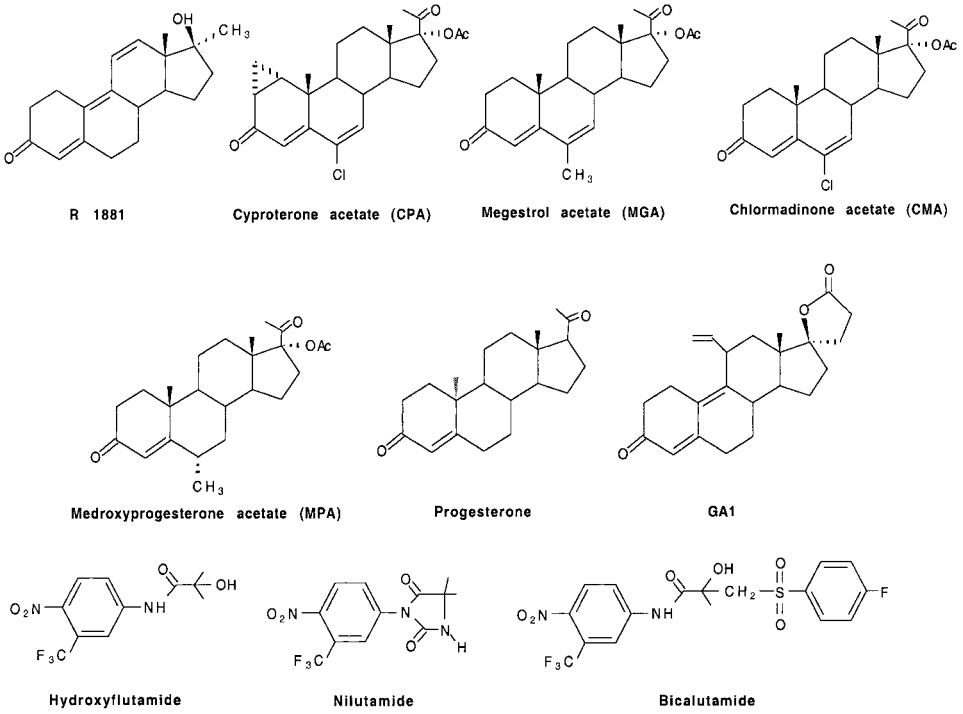

Fig. 1. Chemical structures of steroidal ligands.

(PerkinElmer Life Science). The pSG5-hAR was created in the laboratory by Dr. Patrick Balaguer (Montpellier, France) according to the protocol described by Shemshedini et al. (1991). pFC31Luc, which contains the murine mammary tumor virus (MMTV) promoter driving the luciferase gene (Gouilleux et al., 1991), was obtained from H. Richard-Foy (LBME, Toulouse, France). For transient transfection, all plasmids were purified with nucleobond-AX cartridges (Macherey-Nagel, Hoerdt, France).

Androgen Binding and Competitive Inhibition Assays. Monkey kidney COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Cergy Pontoise, France), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), in a humidified atmosphere containing 5% CO_2. The apparent binding affinity of the wt-AR and mutant ARs was determined by plating 1.5×10^5 cells in 12-well tissue culture plates. After 8 h, the cells were transiently transfected with 50 ng of pSG5-hAR wt or mutant and 200 ng of pCMV-β-galactosidase using the calcium phosphate coprecipitation method. After overnight incubation, precipitates were removed and replaced by fresh Dulbecco's modified Eagle's medium with 3% dextran-coated charcoal fetal calf serum (DCC-FCS). After 48 h, transfected cells were incubated for 2 h at 37°C with increasing concentrations of [^{3}H]R1881 from 0.05 to 4 nM for total binding. Nonspecific binding was measured in parallel incubations containing an additional 1000-fold molar excess of radioinert ligand. Aliquots of total and free-labeled steroid were taken after the binding reaction. The plates were put on ice for 15 min and labeling medium was removed. The cells were washed three times with 1 ml of cold phosphate-buffered saline, and harvested in 300 μl of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM CDTA, 10% glycerol and 1% Triton X-100). After washing as described above, specific radioactivity was determined on 100 μl of lysate. Specific binding was determined as the difference between total and nonspecific counts. The dissociation constants (K_d) and the maximum androgen-binding sites (B_{max}) were derived from Scatchard plots.

The apparent inhibition constant (K_i) for CPA was determined using COS-7 cells transiently transfected with wtAR or G708AAR as described above. Cells were labeled for 2 h with 1 nM [^{3}H]R1881, in the presence or absence of increasing concentrations of CPA. Apparent K_i values were determined using a Microsoft Excel macro provided by Dr. W. Koerner (Augsburg, Germany).

Immunoblot Analysis. Immunoblotting was performed in the COS-7 cell line as described previously (Lobaccaro et al., 1999). Briefly, 1.5×10^6 cells were plated on 10-cm dishes and transfected with 8 μg of plasmid. Sixteen hours after transfection, the cells received fresh medium with 10^{-8} M R1881 or vehicle and were cultured for an additional 30 h before harvesting. The cells were washed twice with phosphate-buffered saline and lysed in the presence of a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were subjected to electrophoresis and gels were blotted onto Hybond membrane (Amersham Biosciences, Paris, France). For AR detection, Western blotting was performed using the rabbit polyclonal antibody N-20 directed against a peptide corresponding to amino acids 2 to 21 mapping at the N terminus of the human AR (Santa Cruz Biotechnology, Tebu, France). For detection of β-actin, membranes were incubated with the polyclonal antibody developed in rabbit using the C-terminal actin fragment (Sigma). Blots were stained using a chemiluminescent detection system (Pierce, Interchim, Montluçon, France).

Limited Proteolysis Assays. Expression plasmids (1 μg) (wt AR or G708A AR mutant) were transcribed and translated with the TNTT7 quick-coupled transcription/translation system (Promega, Charbonnieres, France) as described previously (Georget et al., 2002) in the presence of [^{35}S]methionine (1000 Ci/mmol; ICN, Orsay, France) for 2 h at 30°C. Five microliters of ^{35}S-labeled receptor synthesized in vitro was preincubated for 30 min at 37°C with 0.5 μl of vehicle or ligand. Limited proteolysis was performed by the addition of 5 μl of various concentrations of trypsin (final concentrations, 0.25 and 50 μg/ml). Incubations with protease were conducted for 10 min at 27°C and stopped by addition of 10 μl of SDS sample buffer and cooling in ice. Samples were boiled for 5 min. The products of proteolysis were separated on a 0.75-mm thick 12% SDS polyacrylamide gel. After electrophoresis, the gels were washed in distilled water and vacuum-dried for 20 min. Gels were exposed to a Fujix film imaging plate for 1 h and to autoradiography overnight.

Transfection and Luciferase Activity Assay. CV-1 cells were cultured in the same medium as COS-7 cells. The cells were seeded in 24-well plates (10^5 cells per well) and transfected 8 h after using calcium phosphate with 25 ng of wt-AR or mutant ARs, 125 ng of pCMV–β-galactosidase, and 500 ng of pFC31. The precipitate was removed after 16 h, and the cells were maintained in fresh Dulbecco's modified Eagle's medium with 3% DCC-FCS with vehicle alone or various ligand concentrations. After 30 h, the cells were lysed by 300 μl of lysis buffer described above. The luciferase activity was measured by the reaction of lysate with the luciferin solution: 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, 20 mM Tricine, pH 7.8, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, and 1 mM EDTA. Luciferase activity was measured on an Amersham Biosciences luminometer. β-Galactosidase activity was determined to control the efficiency of each transfection. At least three independent assays were done in duplicate.

Model Building. The atomic coordinates of the androgen agonists, DHT and R1881, were extracted from the crystallographic data files [Protein Data Bank (http://www.rcsb.org/) (Bernstein et al., 1977; Berman et al., 2000) ID codes 1i37 and 1e3g, respectively (Matias et al., 2000; Sack et al., 2001)]. Modeling of the required mutants was performed using Insight II software (Insight II ver 2.7; Accelrys Inc., San Diego, CA) as the main program, and some optional modules. We constructed the antagonist CPA (Fig. 1) from the heavy DHT structure with the ligand design module. Energy was minimized using the Discover module until the deviation root mean square was below 0.01 kcal/mol. Ligand docking was initially carried out by superposition of the antagonist steroid structure onto the DHT agonist steroid. Hydrogen bonds between residues Q711 and R752 and the C3-ketone group of the A-ring were preserved. The ligand-receptor complex was then submitted to energy minimization using the Discover module as described above. We applied the consistent valence force field and the conjugate gradient algorithm together with a cut-off distance of 50 Å. Minimization was performed for 3000 iterations or until the maximum derivative was less than 0.1 kcal/Å. AR mutants were generated with the biopolymer module, and the ligand-AR mutant complexes submitted to energy minimization as above.

Results

Ligand Binding to Mutant hARs. Several point mutations were introduced in wt hAR at position 708 on helix H3 of the LBD. The glycine residue was replaced with the hydrophobic amino acid alanine or valine or with an aromatic phenylalanine residue. Verification of expression levels in transfected COS-7 cells revealed an absence of stabilization of hAR mutants G708V and G708F in the presence of the agonist R1881 (Fig. 2). COS-7 cells transfected with wt or mutant hAR expression vectors were incubated with varying concentrations of [^{3}H]R1881 or [^{3}H]R1881 plus a 100-fold excess of unlabeled R1881. The specific binding sites and the apparent dissociation constants (K_d) of the ligand were determined by Scatchard analyses (Fig. 3). R1881 bound to wt hAR with an affinity of 0.25 ± 0.05 nM ($n = 3$), which is in the same range as those previously reported for COS-7 cells (Veldscholte et al., 1990; Ris-Stalpers et al., 1993). The substitution of G708 by alanine did not significantly alter the hAR ligand-binding capacity. G708V exhibited much lower

affinity for R1881, with a K_d of 3.55 ± 0.5 nM, and no affinity could be determined for the binding failure of G708F. These results suggest that the synthetic agonist R1881 is well accommodated in the LBP of the G708A mutant as well as in the wt hAR.

Transactivation Properties of the wt and Mutant hARs. Transcriptional activity was measured in CV1 cells transfected with an hAR expression vector and a MMTV-Luc reporter gene (MMTV-Luc). Activity was measured over a range of ligand concentrations. Data are represented as fold induction of luciferase activity determined relative to the activity in absence of R1881 (Fig. 4). G708A retained the ability of the wt hAR to stimulate transcription in response to R1881. The G708V mutation dramatically altered the transcriptional activity compared with G708, and G708F failed to transactivate the androgen-regulated gene.

Effect of CPA on [^{3}H]R1881 Binding to hAR and G708A. Binding experiments were performed to test the ability of CPA to compete with [^{3}H]R1881 for the hormone binding sites on the hAR and G708A mutant. The displacement curve of CPA is shown on Fig. 5. The K_i values (concentration of competitor required to reduce the specific radioligand binding by 50%) were equal to 3.10^{-8} M for hAR and 10^{-7} M for G708A, respectively. The results indicate that the G708A mutation slightly altered the binding affinity for CPA.

Effect of Antiandrogens on the Transcriptional Activity of wt hAR and G708A Mutant. We tested the agonist and antagonist activities of antiandrogens with G708A compared with wt hAR. The steroidal antiandrogens were CPA, MGA, and CMA, and the nonsteroidal antiandrogens were hydroxyflutamide, nilutamide, and bicalutamide (Fig. 6). Progesterone (Fig. 7) and compound GA1 (Fig. 8), an antimineralocorticoid recently demonstrated to be a pure androgen (Nirdé et al., 2001), were also tested.

The agonist activities were measured with increasing concentrations of antiandrogen. Values are represented as percentage response, with 100% activity defined as the activity achieved with 10^{-8} M R1881. The antagonist CPA, which usually displays a partial agonist activity for wt hAR (40% at 10^{-6} M, Fig. 6A, a), did not exhibit any activity for G708A. MGA and CMA revealed a weak agonist activity (10% at 10^{-6} M) for the mutant (Fig. 6A, a). Conversely, partial agonist activity was observed with 20% hydroxyflutamide (Fig. 6B, a). No significant luciferase activity was measured in the presence of nilutamide and bicalutamide.

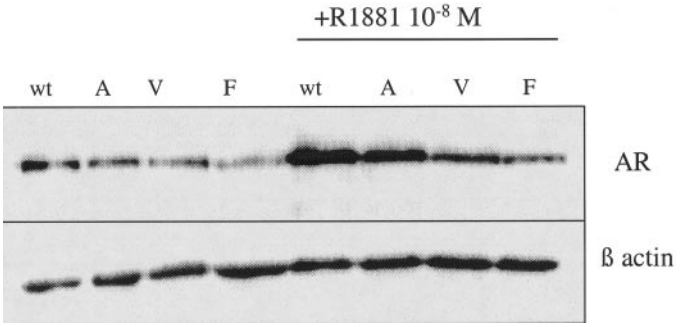

Fig. 2. Expression levels of different variants of G708 mutated form. COS-7 cells were transfected with expression vectors and whole extracts were analyzed by Western blotting using polyclonal antibodies against hAR and β-actin to control equal protein loading.

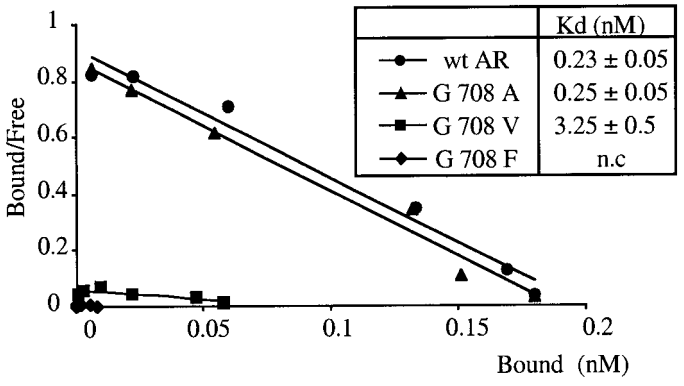

Fig. 3. Apparent equilibrium binding affinities of wt hAR and G708 mutants for [^{3}H]R1881 measured at 37°C in transiently transfected COS-7 cells. The dissociation constants (K_d) and the maximum androgen-binding sites (B_{max}) were derived from Scatchard plots. Results represent the means of at least two independent determinations performed in triplicate. n.c., not calculable under our experimental conditions.

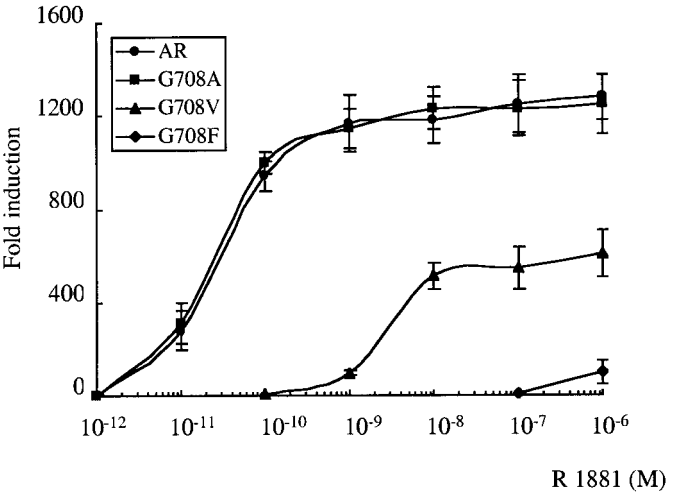

Fig. 4. Transcriptional activity of the wt and mutant hARs. CV-1 cells were cotransfected with vectors expressing receptors, an androgen-regulated gene (MMTV-luciferase) and plasmid for β-galactosidase constitutive expression. The cells were incubated in the presence of increasing concentrations of R1881. Shown is the -fold induction relative to activity determined in the absence of R1881. The data are representative of at least three independent experiments.

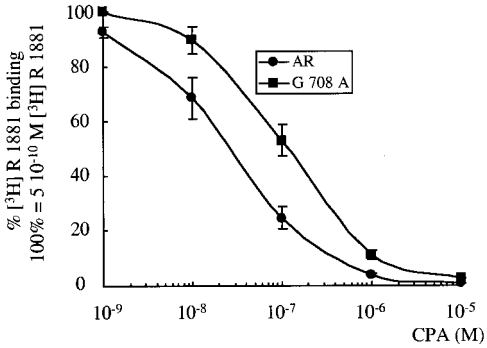

Fig. 5. Competitive inhibition of [^{3}H]R1881 binding to hAR and G708A mutant in COS-7 cells by CPA. COS-7 cells on 12-well plates were transfected with the expression vectors wt hAR and G708A hAR. The cells received fresh medium containing 3% DCC-FCS 16 h after transfection and were maintained for 30 h in this medium. The cells were labeled for 2 h in 1 nM [^{3}H]R1881, in the presence or absence of increasing concentrations of CPA. Data are expressed as percentage of specific binding observed in the noncompeted control. Each point is the mean ± S.E.M. of three separate experiments.

To compare the antagonist activity of antiandrogens on transcriptional activation with hAR and G708A mutant, transfected cells were incubated with different concentrations of antiandrogen in presence of 10^{-10} M R1881. The antagonist potency of the ligands was performed with increasing concentrations of antiandrogen with 10^{-10} M R1881 (values are represented as percent response, with 100% activity defined as the activity achieved with 10^{-10} M R1881). Fig. 6A, b, revealed that 10^{-6} M CPA reduced the transactivation induced by R1881 up to 40% for G708 and totally inhibited the R1881 activity of G708A. The dose-response curves of inhibition with MGA and CMA were similar, and no significant difference was observed compared with CPA with the mutant. G708A induced luciferase activity only up to 40% for 10^{-6} M of hydroxyflutamide (Fig. 6B, b). No antagonist activity was observed with the mutant for nilutamide, and no change in antagonist activity was found for bicalutamide. These results demonstrate that the G708A mutation was able to transform the partial antiandrogen CPA into a pure antiandrogen and the pure nonsteroidal antiandrogen hydroxyflutamide into a partial agonist.

The antagonism exerted by progesterone was similar at 10^{-6} M with wt hAR and G708A (Fig. 7). G708A exhibited a right shift in the dose-response curve for compound GA1, nearly 2 orders of magnitude compared with wt hAR (Fig. 8). This compound, described as a full agonist on the wt hAR displayed partial antagonist properties on G708A.

Antiandrogen-Induced Conformational Changes of G708 and G708A-hARs. We investigated whether the modifications in the antagonist properties observed with G708A were the result of an inappropriate conformation of the receptor. Limited proteolysis assays were performed with the wt and mutant receptors incubated with antiandrogens (Fig. 9). CPA (10^{-5} M) stabilized a wt hAR conformation, providing a 35-kDa band (inactive conformation), whereas an important 29-kDa band (active conformation) appeared at the same concentration. Conversely, we observed that G708A was protected in a major 35-kDa resistant fragment. We also observed that hydroxyflutamide, nilutamide, and bicalutamide incubated with wt hAR allowed the detection of the inactive 35-kDa fragment. Nilutamide and bicalutamide induced the resistance of G708A in a 35-kDa fragment but a conformational change in the presence of 10^{-5} M of hydroxyflutamide was observed with the protection of the 29-kDa fragment.

Ligand Docking. The two structures of the AR-LBD, in complex with the natural ligand DHT and the synthetic agonist R1881, revealed that the hAR-LBP is outlined by helices H3, H5, H7, H11, and H12; the β turn and loops 6, 7 and 11, 12 (Matias et al., 2000; Sack et al., 2001). It is limited by 19 amino acids that are mostly hydrophobic, with the exception of Asn705, Gln711, Arg752, Glu783, and Thr877. G708 is located in helix H3 and has direct interactions with the ligands. The G708A-LBD mutant was generated based on

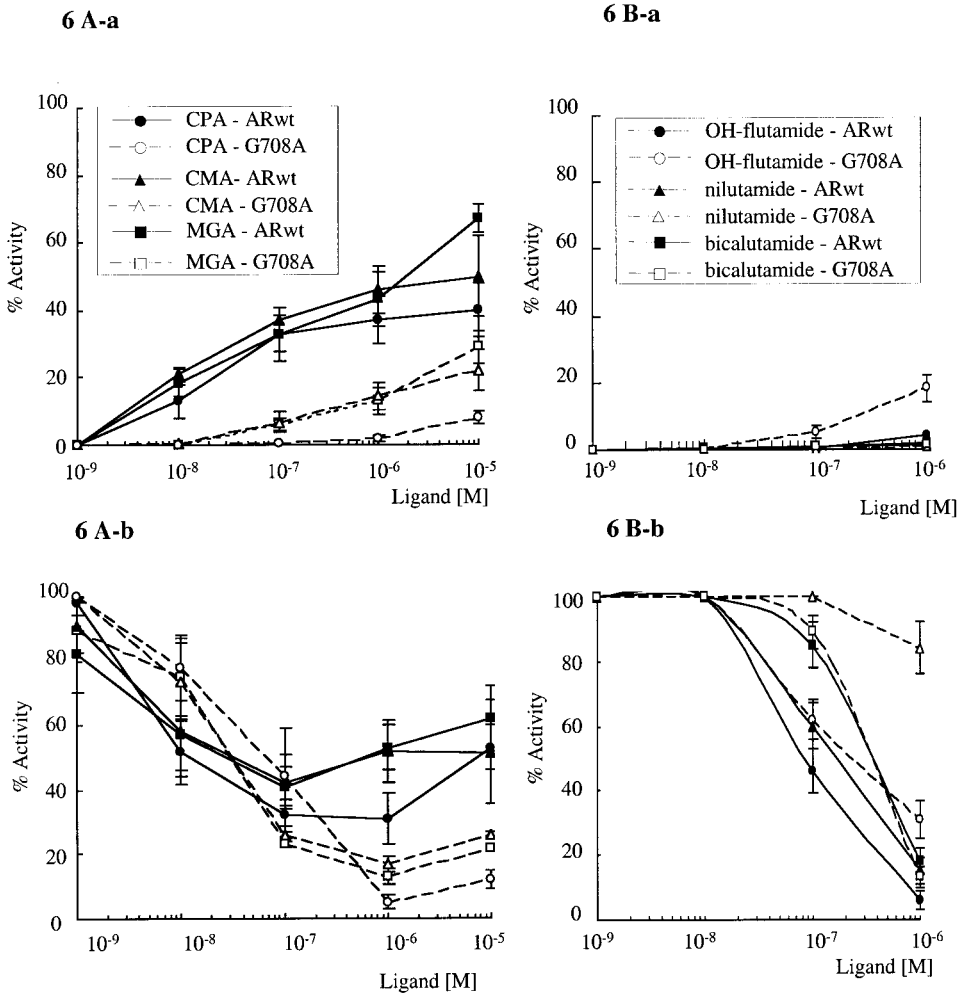

Fig. 6. Agonist and antagonist effects of antiandrogens on transcriptional activities of hAR and G708A mutant. CV-1 cells transfected with AR expression vectors and androgen-dependent reporter gene (MMTV-luc) were incubated in the presence of increasing concentrations of antiandrogen alone or in competition with 10^{-10} M R1881. A, steroidal antiandrogens (CPA, MGA, CMA): a, agonist activity; b, antagonist activity. B, nonsteroidal antiandrogens (hydroxyflutamide, nilutamide, bicalutamide): a, agonist activity; b, antagonist activity. Luciferase activities are expressed relative to AR activity with 10^{-8} M R1881 alone for agonist activity or with 10^{-10} M R1881 alone for antagonist activity. Values represent the means ± S.E.M. from three or more experiments.

the existing AR-LBD structures, and ligand docking in either the wt hAR or G708A mutant LBPs was initially carried out by superposition of the antagonist steroid CPA onto the DHT or R1881 agonist steroids. This reveals that the synthetic agonist R1881 and the natural ligand DHT are well accommodated in the LBP of G708A. In contrast, it is likely that the partial agonist CPA will adopt a slightly different orientation because of the presence of a cyclopropyl ring in the A-ring and a bulky chlorine on the 6-position of the B-ring. Indeed, steric constraints were observed between the cyclopropyl ring and Leu707 (helix H3, 2.89 Å) and between C6-Cl and Met787 (helix H7, 2.20 Å) and Phe764 (β turn, 2.90 Å) (Fig. 10A). Moreover, the acetate and acetyl moieties in C17 were in close contact with Leu701 (helix H3, 1.15 Å), Leu880 (helix H11, 2.65 Å) Thr877 (helix H11, 1.35 Å), and Phe891 (loop H11,H12, 2.8Å (Fig. 10B). Minimization of the various complexes, as described under *Material and Methods,* reveals that major rearrangements of the LBP side chain residues allow docking of CPA into the agonist form of AR LBD.

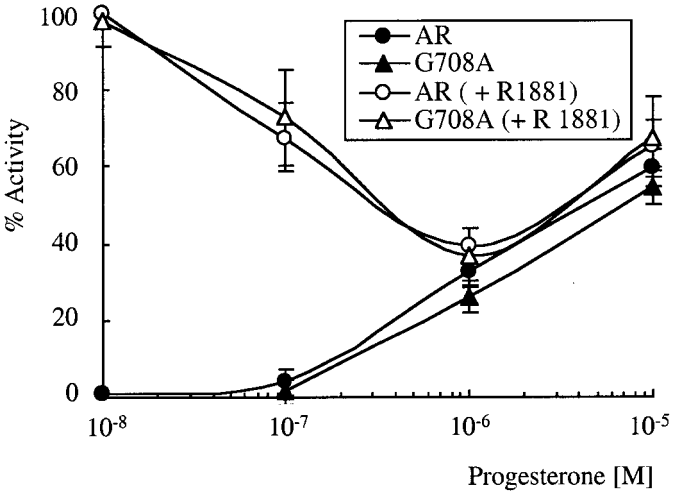

Fig. 7. Agonist and antagonist effects of progesterone on the transcriptional activities of hAR and G708A mutant. Experiments were performed with 10^{-8} to 10^{-5} M progesterone in combination with 10^{-10} M R1881. Luciferase activities are expressed relative to AR activity with 10^{-8} M R1881 alone for agonist activity or with 10^{-10} M R1881 alone for antagonist activity. Values represent the means ± S.E.M. from three or more experiments.

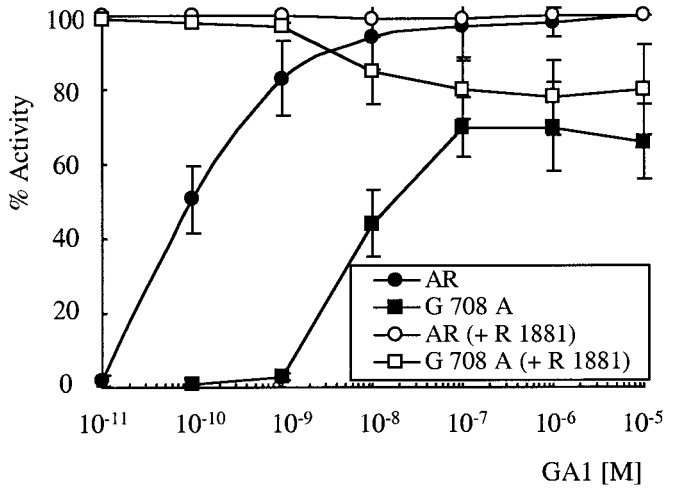

Fig. 8. Agonist and antagonist effects of compound GA1 on the transcriptional activities of hAR and G708A mutant. Experiments were performed with 10^{-11} to 10^{-5} M GA1 in combination with 10^{-10} M R1881. Luciferase activities are expressed relative to AR activity with 10^{-8} M R1881 alone for agonist activity or with 10^{-10} M R1881 alone for antagonist activity. Values represent the means ± S.E.M. from three or more experiments.

Discussion

As described recently (Nirdé et al., 2001), the region surrounding Gly708 in the hAR seems to be crucial for the stabilization of the active hAR conformation. The sequence alignment of the hAR revealed that this glycine residue is well conserved, as it is in human progesterone receptor and human glucocorticoid receptor. This suggests the key role of this amino acid in the functionality of nuclear receptors, although it should be noted that hMR and hER possess an alanine at the corresponding position. Furthermore, an AR gene mutation was detected by single-strand conformation polymorphism in codon 708, leading to an amino acid substitution Gly708A (Hiort et al., 1994; Albers et al., 1997). This point mutation causes a partial androgen insensitivity syndrome clinically characterized by undervirilization in 46XY male patients.

We previously showed (Auzou et al., 2000) that the Ala773Gly substitution in hMR was critical for generating agonist mineralocorticoid activity in the 11β-substituted spirolactones synthesized in our laboratory and typically known as antimineralocorticoids (Faraj et al., 1990). The same compounds were recently described as potent androgen agonists (Nirdé et al., 2001), and the importance of Gly708 and its surrounding region in accommodating these 11β substituents was demonstrated. The results reported in the present study define the size of the side-chain residue that can be accommodated at position 708 without altering AR functions. Steric hindrance induced by residues such as Val and Phe altered or abolished the binding of the steroidal agonist R1881. In contrast, the K_d values of G708 and G708A for R1881 were identical. Similarly, the transactivation curves of G708 and G708A for R1881 were identical, whereas G708V induced a greater shift toward higher concentrations, and no R1881-mediated transactivation function was detectable for G708F even at micromolar concentrations. In conclusion, our study reveals that a substitution of Gly708 with alanine can

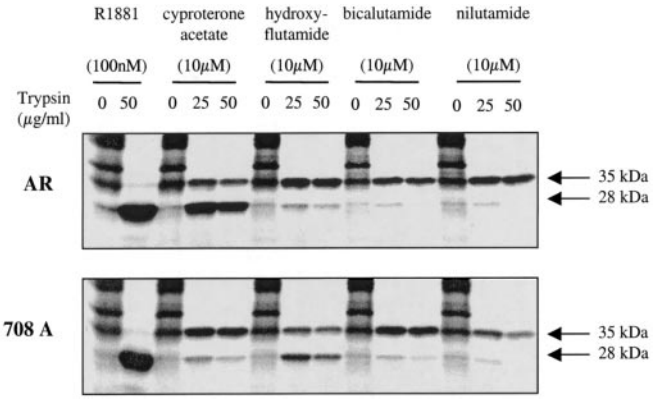

Fig. 9. Antiandrogen-induced conformation changes of hAR and G708A mutant. Incubation of in vitro-produced AR with cyproterone acetate (10^{-5} M), hydroxyflutamide (10^{-5} M), bicalutamide (10^{-5} M), or nilutamide (10^{-5} M) was followed by a treatment with trypsin (25 and 50 μg/ml) for 10 min at 27°C, Trypsin-digested products were separated by SDS-polyacrylamide electrophoresis and visualized after autoradiography.

be tolerated without the loss of agonist binding affinity, whereas it seems that the presence of a valine or a phenylalanine at position 708 reduces agonist binding partially or completely. Structural analysis indicates that there is not enough room for bulky residues at position 708.

The antiandrogen CPA possesses an A-ring with 1,2 cyclopropane moiety, a 6-chlorine atom on the B-ring, and C17 substituents that differ significantly from that of R1881; CPA displays partial agonist properties on the wt hAR. Competitive inhibition of [^{3}H]R1881 binding to wt hAR and G708A induced by CPA in COS-7 cells revealed a slight shift of the dose-response curve with K_i values ranging from 3×10^{-8} to 10^{-7} M. Moreover, the Gly708Ala mutant transformed the partial steroidal androgen CPA into a pure antiandrogen. This result was confirmed by proteolysis assays, which showed the major 35-kDa resistant fragment with the mutant G708A corresponding to the inactive conformation. The active conformation (29 kDa) was not observed for the mutant. The superposition of CPA on either DHT or R1881 in complex with AR-LBD revealed very close contacts between the CPA C6-chlorine atom and Met787 (H7) and Phe764 (s1), with interatomic distances of 2.2 and 2.9 Å, respectively. In addition, the cyclopropane ring was at a distance of 2.89 Å from Leu707 (H3). Therefore, the binding of CPA to wt hAR or G708A requires some structural adaptations. Indeed, our modeling study indicates that CPA can be accommodated within the wt hAR or G708A LBPs after major side chain conformational changes. Surprisingly, in the resulting models, it seems that the 17α acetyl and acetate moieties do not directly preclude the active (holo) conformation of helix H12. Therefore, the "passive antagonism" first described for the ERβ antagonist 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (Shiau et al., 2002) could account for the partial agonist character of CPA. In this recently described mechanism, the structural basis of antagonism does not rely on the presence of a bulky extension on the antagonist ligand but rather on the production of suboptimal side chain conformations of residues involved in the interaction with helix H12 in its active conformation. The proper hydrophobic binding surface for the holo-helix H12 is not generated; therefore, the surface involved in the recruitment of coactivators and composed of helices H3, H4, and holo-H12 is destabilized.

To compare the effects of these substituents on the steroidal scaffold, we tested MGA and CMA, both of which lack the cyclopropane ring of CPA but possess a methyl (MGA) or a chlorine (CMA) at position 6 of the B-ring, as well as 4,5–6,7 double bonds and the same 17α-acetyl moiety on the D-ring. Similar partial agonist activity with wt hAR and antagonist activity with the mutant G708A were observed for both ligands (Figs. 6A, a and b). These results strongly suggest the role not only of different C-6 substituents but also of the 4,5–6,7 double bonds on the conformation of these three compounds, which enhance the rigidity of these molecules and hamper the correct positioning of helix H12. This result was further supported by the weak antagonist activity of progesterone deprived of this 4,5 double bond and 17α-acetyl moiety. In this last case, no pure antagonist activity of progesterone on G708A mutant compared with CPA, MGA, and CMA was observed at 10^{-6} M, and the transactivation curves revealed an increase in the agonist activity at higher concentrations. The absence of this 4,5 double bond was also observed with medroxyprogesterone acetate, an analog of MGA that possesses a 6α-methyl group and only one double bond (4,5) on the A-ring. A recent report revealed the agonist activity of this compound on CV-1 cells compared with CPA, as we observed herein (Kemppainen et al., 1999). The orientation of this 6α-methyl group, which differs from 6-CH_3 on MGA, and the presence of only one 4,5 double bond probably confirm the integrity of the conformation of medroxyprogesterone acetate and its agonist activity.

Compound GA1, which is characterized by a 17γ-lactone ring, a C-11β vinyl hydrophobic substituent, and 4,5, 9,10, and 11^1,11^2 double bonds, displayed partial agonist activity when acting through the wt hMR and was almost a full agonist with hMR Ala773Gly (Auzou et al., 2000). We recently showed that this derivative exhibited an agonist activity without antagonist activity on hAR (Nirdé et al., 2001). In the present study, we showed that the substitution of Gly708 by Ala induced a greater shift in the dose-response curve toward higher concentrations and produced lower transactivation efficiency in response to compound GA1 (2 orders of magnitude); nevertheless, a weak antagonist activity was observed. This result reveals that hydrophobic contacts between the C-11β substituent and the already described Gly708, Trp741, and Met895 residues are probably maintained, as well as the hydrophobic contacts with the

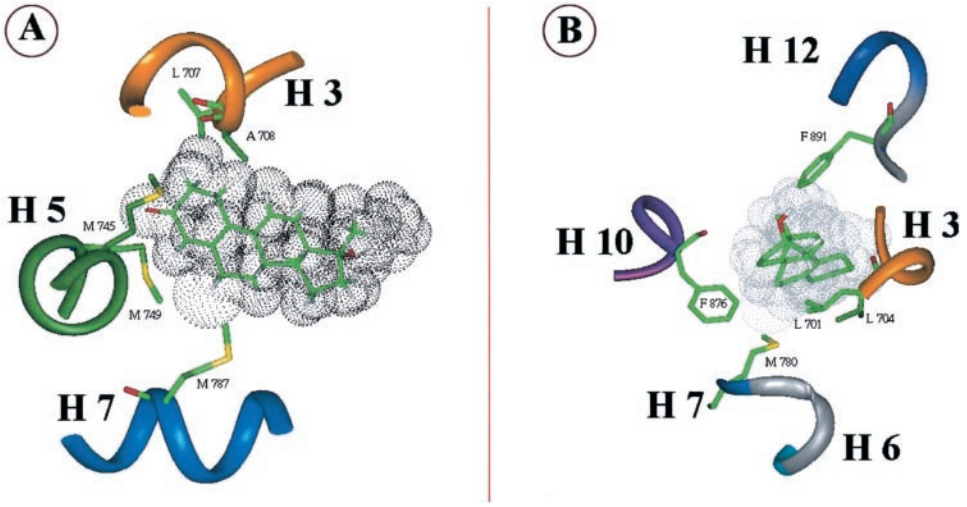

Fig. 10. Ligand docking (R1881 and CPA) of the hAR-ligand-binding pocket. A, close-up view of the A-ring interaction of R1881 (agonist, stick representation) and CPA (antagonist, dot representation) with L707, A708, and M787. B, close-up view of the D-ring interaction of R1881 (stick representation) and CPA (dot representation) with L701, F876, F891, and M895.

17γ-lactonic ring (Leu701, Leu780, Val889, and Phe891) and the hydrogen bonds with Asn705 and Thr877.

Among the nonsteroidal antagonists, we observed a partial agonist activity for the mutant with hydroxyflutamide that was confirmed by proteolysis assays. The antagonist activity of bicalutamide was conserved, but nilutamide lost it. The docking of hydroxyflutamide has been described previously (Poujol et al., 2000; Marhefka et al., 2001) and revealed a hydrogen bond between the carbonyl group of Asn705 and the α-hydroxyl moiety. In addition, the oxygen atoms of the nitro moiety are hydrogen-bonded to Arg752 and Gln711. Additional anchoring contacts induced between this small molecule and other residues, not yet available, may help to explain the partial agonist activity of hydroxyflutamide with the Gly708Ala mutant.

In conclusion, this work confirms the crucial importance of the region surrounding Gly708 in the anchoring of steroidal hAR agonists and antagonists. Our study demonstrates that only one mutation was able to influence the agonist versus antagonist activity of steroidal ligands. We propose that CPA, MGA, and CMA define a subfamily of AR antagonists that, in contrast to classic antagonists, act through a passive mechanism, thereby suggesting new orientations for the design of selective androgen antagonists.

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